Bone Mechanics HANDBOOK

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Molecular Biology Techniques to Measure Skeletal Gene Expression

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3.1 Introduction

The goal of this chapter is to provide the biomechanical engineer with a description of the fundamental tools and procedures that are currently used to access the quality and quantity of genes made by specific tissues and cells. Genes can be controlled at many levels, as shown schematically in Fig. 3.1. The control of gene expression begins in the nucleus where a combination of DNA sequences (*cis* acting) and proteins (transcription factors, or *trans* acting) interact in the nucleus to make (transcribe) RNA, which then transits to the cytoplasm. By using the ribosome machinery (made of 18S and 28S RNA embedded in proteins) (see Fig. 3.2), the mRNA is translated into proteins that are either used within the cell or secreted and function outside the cell. This chapter will focus exclusively on the analysis of mRNA expression. The methods described are designed to compare the quantity (amount) and quality (specific type of gene) of mRNA activation in control and experimental situations. Thus, the techniques described are used to evaluate the response of a cell or tissue to environmental influences such as biomechanical stress specifically at the "mRNA level." By examining the nature of the RNA that is altered one can extrapolate the corresponding changes in protein expression and develop a framework for understanding the molecular

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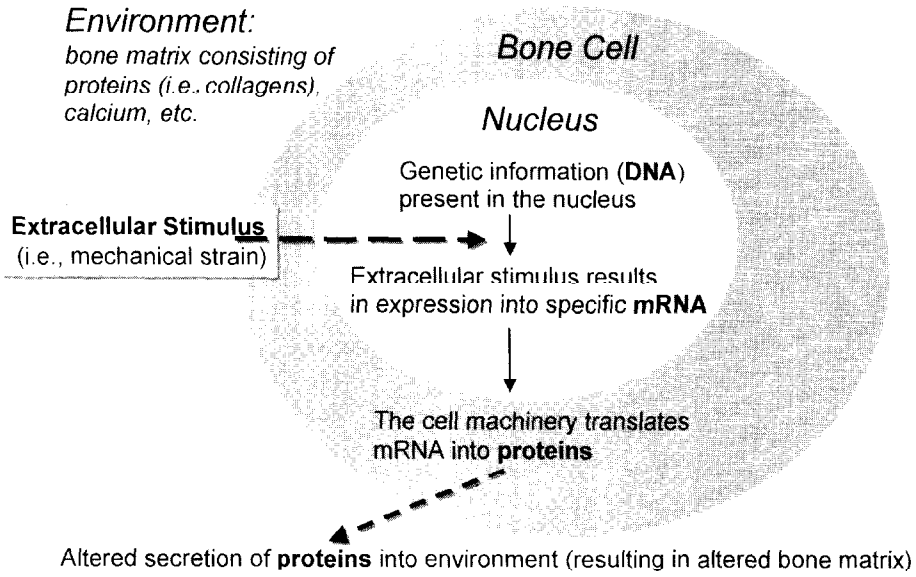

FIGURE 3.1 Schematic representation of gene expression response to extracellular stimuli. This diagram shows the pathway mRNA activation starting in the nucleus. Extracellular stimuli alter this process, leading to changes in the amount and quality of genes (mRNA) expressed. This in turn leads to changes in protein expression that results in an altered bone matrix.

events that accompany changes in skeletal structure and function. Altered protein production results in altered bone matrix composition that can, in turn, affect bone elasticity.

3.2 Examination of Known (Previously Identified) Genes

3.2.1 Northern Blotting

One of the earliest methods that was used to assess gene expression is Northern blotting (Fig. 3.2). In this procedure RNA is extracted from tissues or cultured cells (see Chapter 2) using denaturing agents such as guanidine isothiocyate. Proteins are removed from extracted mixture using organic reagents such as phenol and RNA is concentrated by precipitation with alcohol. In this modern era numerous "kits" are available for RNA isolation, which have all necessary reagents prepacked ready for use. For the "home-made" protocols, several excellent manuals have been written and should be referred to for details of this and other molecular procedures.[1,2] The quantity of RNA extracted is determined by its absorbence of ultraviolet (UV) light at Å260. The quality of RNA is tested by electrophoresis of the RNA through a denaturing agarose gel resulting in separation of the RNA that is expressed. By including a small quantity of ethidium bromide in the samples, the separated RNA can be visualized under shortwave UV light and appears as two bright bands representing the 28S and 18S ribosomal RNA species. The messenger RNA (mRNA or RNA that gets expressed into protein, which together with ribosomal RNA represents what is called total RNA) is not perceptible in the gel and needs further processing for visualization. For easier handling, RNA is transferred from the gel to a solid support or membrane that is made of nitrocellulose or nylon or a mixture of both. The "blotting" refers to the transfer technique; typically RNA is released from the gel by capillary action by placing it next to the membrane. After blotting the RNA is immobilized onto the membrane by UV cross-linking or by heating under a vacuum. The blot is then immersed in a solution that contains a specific gene fragment previously labeled with the isotope P^{32}; this is referred to as the probe. The basis for Northern blotting is that specific probes will hybridize to complementary RNA sequences on the membrane, thus locating any gene of interest (lock-key principle). After washing away unbound probe the blot is exposed to X-ray film and specific mRNA bands are visualized.

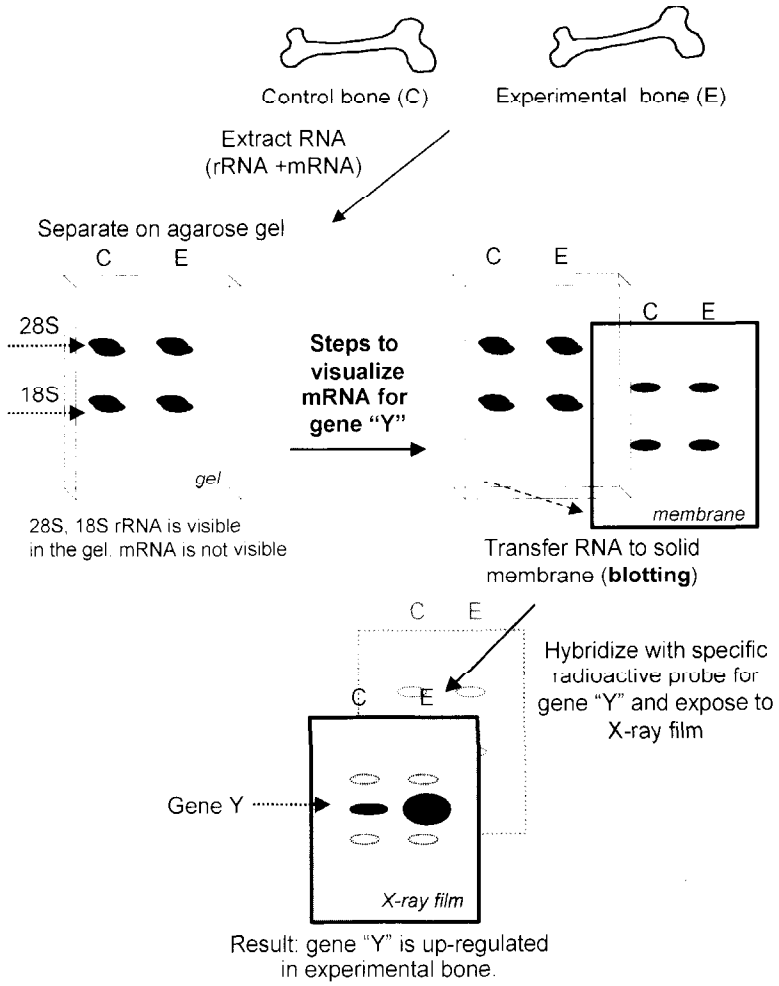

FIGURE 3.2 Northern blotting. In this technique RNA is separated by gel electrophoresis and transferred to a membrane for detection of specific mRNAs. This is done by labeling a specific gene (cDNA) using radioactive nucleotides (typically α-deoxy CTP^{32}) and "random priming." In this procedure short (six base pair) primers are added to the denatured cDNA (see Fig. 3.3) and through the action of the DNA polymerase (see Figs. 3.3 and 3.5) the radioactive nucleotide is incorporated into a new DNA strand thereby making the cDNA radioactive. The labeled cDNA is then used to detect a specific gene, resulting in a specific, quantitative analysis.

Each mRNA has a characteristic length that can be verified using known sized RNA markers on the gel. The intensity of the radioactive bands reveals the relative amount of the mRNA present (see Fig. 3.2). New imaging techniques allow direct quantitation of the mRNA expressed using metal screens coated with material that is sensitive to P^{32}. The exposed screen is then placed directly in an imaging device and the radioactivity present is quantitated using computer software programs.

Using the Northern blotting procedure numerous mRNAs were shown to be altered in the bones of animals subject to spaceflight,[3,4] mechanical unloading,[5,6] or unloading followed by reloading.[7]

3.2.2 Reverse Transcriptase-Polymerase Chain Reaction

One drawback to the Northern blotting procedure is that at least 1 to 20 μg of RNA (depending on the expression level of the gene of interest) is required for each analysis. To examine very low quantities of mRNA, a problem that often occurs in bone material, some investigators have turned to the powerful

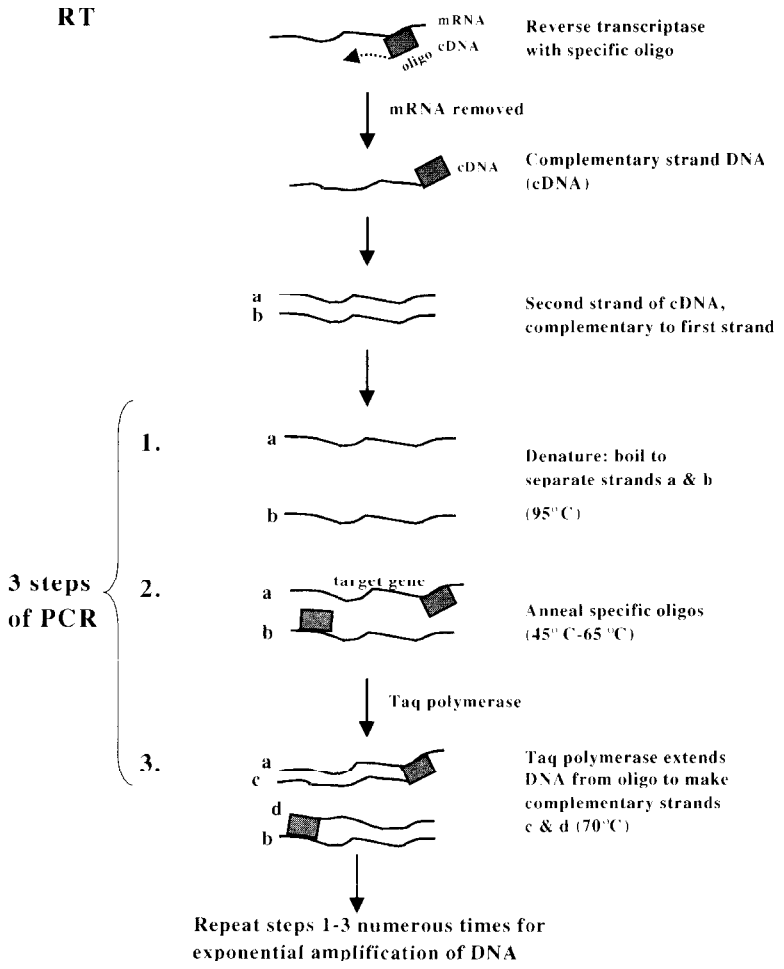

FIGURE 3.3 RT-PCR. This procedure has two parts: (1) a conversion of mRNA to cDNA through the action of RT and (2) amplification of the cDNA using the PCR. Products of the reaction are visualized after separation by gel electrophoresis.

new technique of PCR (polymerase chain reaction) (Fig 3.3). This procedure allows the amplification of minute amounts of material; there are reports of gene detection using a single cell.[8,9] In this method RNA must first be converted to DNA using the enzyme reverse transcriptase (RT). The resulting copy of RNA is called a cDNA and is used as a template for the PCR reaction. By using the enzyme polymerase that was originally isolated from bacteria that live at temperatures up to 70°C (*thermus aquaticus*), DNA can be amplified specifically and exponentially by repeated incubations at various extremes in temperatures. The specificity of the PCR reaction relies on the construction of two short pieces of DNA called oligonucleotides (oligos) that have complementary sequences in the target gene of interest. Different temperatures are used for the three following steps of the PCR process: (1) denaturing the DNA (>95°C); (2) annealing (binding) of the oligo to the target DNA (~45 to 65°C depending on sequence and length of oligos used); and (3) extending the DNA by the action of the polymerase (~70°C) (see Fig. 3.3). The PCR product usually can be visualized directly on agarose gels by staining with ethidium bromide. Every cell contains genomic DNA coding for all genes in the body. It is essential that RNA preparations are not contaminated with genomic DNA; if it is present, it can be amplified and will mask the intended target, which is the mRNA (see Fig. 3.1). DNA can be removed by either (1) treating the RNA preparation with DNase or

(2) designing oligonucleotides that "span an intron" (see Chapter 4). Introns are sequences in the genomic DNA that are not found in mRNA. If they are amplified by PCR, they are detected because they are much larger than the product predicted from the mRNA sequence. One way to "test the system" is to perform the RT-PCR reaction in the absence of RT; mRNA cannot be amplified unless it is first converted to cDNA and no products should be observed.

Many groups have been successful applying this mRNA analysis procedure. Using RT-PCR, changes in *c-fos* (a transcription factor) mRNA expression have been detected in osteoblasts subject to centrifugation in a manner that simulated a space shuttle launch ($3g$).[10] Similarly, when osteocytes were isolated and stretched using a special device that deformed a petri plate with 2000 to 4000 μE, a notable increase of *c-fos* and COX-2 (enzyme that makes prostaglandin) was detected as early as 15 min after treatment.[11]

Some mRNAs are expressed at very low amounts in the cell. For even greater sensitivity, the PCR product (sometimes called an amplicon) is transferred to a solid support or membrane and analyzed in a fashion that is similar to Northern blotting. The membrane containing the amplicon is hybridized to radioactively labeled probes corresponding to the amplified gene, and the amount of hybridization is measured using X-ray film or image screens described in the previous section (see Fig. 3.2).

One drawback to the RT-PCR procedure is that it is semiquantitative in nature. More quantitative procedures based on RT-PCR have been developed using "mimics." In this case, target genes of interest are modified so that when used as a template for RT-PCR they generate a product with a different size than the normal gene. By adding known amounts of the mimic to the PCR reaction, one can estimate the amount of material required to compete with the normal gene and obtain a more accurate estimate of the amount of a specific mRNA in a sample. This procedure was recently carried out using RNA extracted from MG-63 cells (human osteoblast-like cells) (see Chapter 2) and mimics to the bone matrix proteins type I collagen, alkaline phosphatase, and osteocalcin. All three matrix mRNAs were decreased in cells subject to microgravity.[12]

3.2.3 *In Situ* Hybridization

The aforementioned methods of measuring the type and amount of mRNA rely on extracting mRNA from tissues and cells prior to the analysis. One drawback to these methods is that, if a tissue is heterogeneous and composed of several cell types, there is no way to determine which part of the tissue has changes in gene expression. ISH (*in situ* hybridization) is a method that allows localization of specific mRNAs within a tissue (see Ref. 13 and 14 for detailed methodology). Tissues of interest are fixed in buffered 4% paraformaldehyde and, in general, demineralized prior to embedding for histology; some studies show that undecalcified bone sections can be used,[15] but this is not a commonly used approach. Tissue sections are then hybridized with specific RNA or DNA probes in a manner similar to that described for Northern blotting. Because the P^{32} signal has a low resolution, often P^{33} or S^{35} are substituted for labeling the probes. There is an increasing trend to use nonradioactive labeling protocols as well. The advantage here is that probes can be made well in advance and are not subject to half-life considerations.

In many cases investigators complement ISH with immunohistochemistry (*in situ* detection of the protein of interest). Recently, this combined approach was used to show the upregulation of tenascin-C mRNA and protein in bones exposed to mechanical stimulation.[16] In other cases, several methods of mRNA analysis are additionally combined to give greater confidence in the observed results. A good example is the recent discovery of *N*-methyl-D-aspartate-type receptor in osteoblasts and osteoclasts; a combined approach of RT-PCR, ISH, and immunohistochemistry was used to suggest a novel glutamate signaling pathway in bone.[17]

Recently, the RT-PCR was modified so that it could be performed *in situ* (directly on the tissue section). Specifically, in these studies RNA was reverse transcribed *in situ* and subject to PCR to measure mechanically mediated changes in gene expression in osteocytes. When a mechanical stimulus was applied to bones, elevated levels of collagen mRNA in a turkey model[18] and of β-actin, osteocalcin,

connexin-43, insulin-like growth factor-I (IGF-I), c-fos, and c-jun in a rat model[19] were observed. Even though these findings are a relative assessment of gene activity, this is an exciting new development that combines sensitivity with precise cell localization.

3.2.4 RNase Protection Assay

RNase protection assay (RPA) is a method that combines sensitivity with quantitation of extracted RNA. By using this technique, it is possible to examine multiple genes in a single reaction. A specific RNA probe is made, usually labeled with S^{35} or P^{33}, that has a distinct length. RNA is hybridized to the probe, which is complementary to it and therefore called "antisense." Incubation of the hybrid with RNase destroys single-stranded RNA not hybridized or "protected" by the probe (Fig. 3.4). The resulting material that is protected can be visualized after separation on denaturing acrylamide gels followed by autoradiography

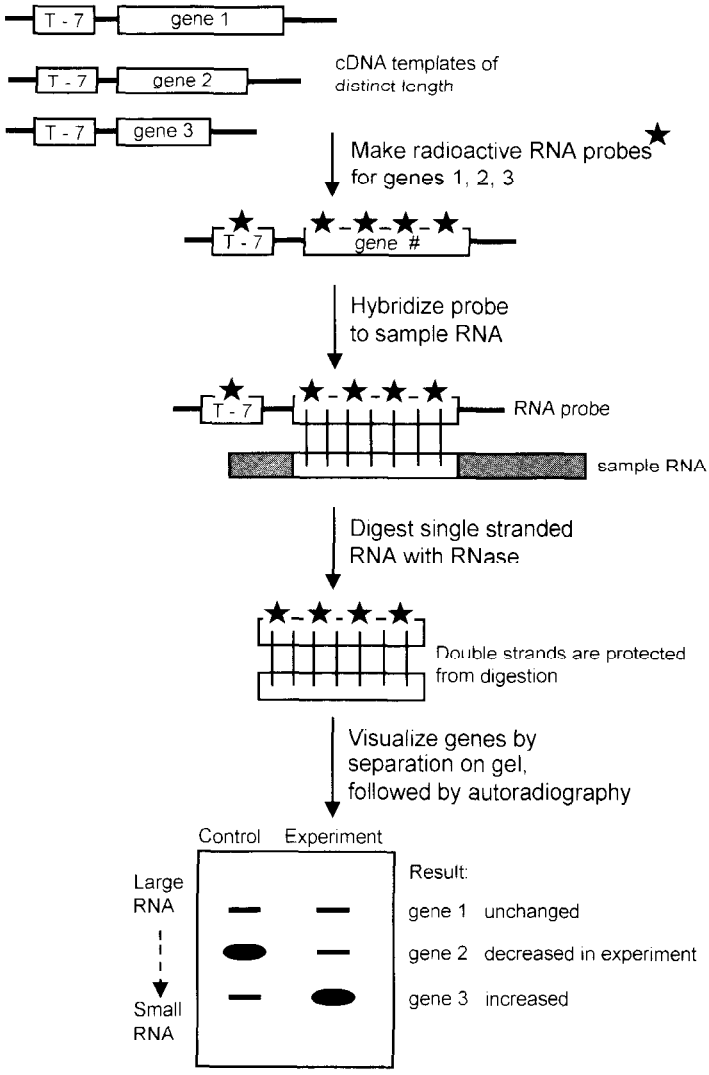

FIGURE 3.4 RPA. Isolated genes are used to make radioactive RNA probes using radiolabeled UTP[P33/S35]. Both sense (same sequence as the mRNA) and antisense (complementary sequence to the mRNA) are used. Only the antisense probe hybridizes and protects the mRNA from the action of added RNase. The protected fragments are visualized by electrophoresis using denaturing acrylamide gels and reveal the relative amount of mRNA present.

similar to Northern blotting. Each gene is identified based on length and quantitated based on its intensity. To date, most RPA kits are geared toward cytokine analysis, but one can generate custom templates using any purified gene of interest.

3.2.5 Array Technology

As more genes are cloned and identified there is a trend to try to examine the expression of numerous genes simultaneously. For this reason array technology was developed, where instead of a dozen genes being analyzed hundreds or even thousands of genes can be examined in a single experiment. The procedure is essentially a modification of the procedures already described. Previously identified genes are amplified and dotted onto membranes in a grid fashion. Instead of labeling specific probes, extracted mRNA is labeled (radioactive or nonradioactive) in an RT reaction (see RT-PCR) and the resulting cDNA is hybridized to the membrane. If an RNA is present in the mixture that corresponds to a gene on the membrane, it will hybridize to it. After washing away nonspecifically bound mRNA, the blot is analyzed as is done for a Northern blot or RPA. Figure 3.5 shows a simplified version of an "array" (i.e., "dot blot"). Current arrays contain hundreds of genes and can be obtained commercially. By using the array technology, analysis of RNA from cells with a nonfunctional estrogen receptor-alpha (ER-α) gene led to the discovery of at least two genes, transforming growth factor-beta (TGF-β) and c-fos, that may be affected by estrogen through ER-α-independent pathways.[20] It must be cautioned here that discovery of differential gene expression using arrays must be confirmed by alternative methods such as Northern, RNase protection, or competitive RT-PCR.

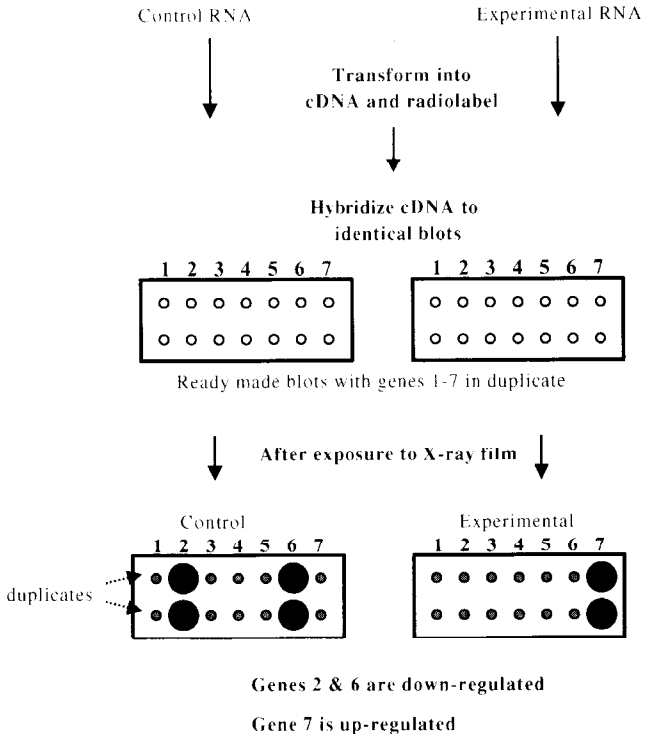

FIGURE 3.5 Arrays. Individual cDNA (genes) are isolated and "dotted" onto a membrane usually in duplicate. mRNA is isolated from control and experimental samples and labeled by incubation with RT, primers, and radioactive nucleotide that will be incorporated into a cDNA (copy DNA). mRNA can also be labeled directly by gentle NaOH digestion (to make it smaller lengths) followed by labeling with T4 kinase and a radioactive nucleotide that will "end label" it such as γ ATP.[32]

3.3 Gene Discovery

3.3.1 Differential Display (DD), Subtractive Hybridization, and Representational Difference Analysis (RDA)

In 1992, Liang and Pardee[21] described a modification of RT-PCR procedure to identify genes that were differentially expressed. In these experiments RNA is extracted and annealed to sets of different oligos (also called primers) that together cover all expressed genes. The oligos are first used to make cDNA using the enzyme RT and then for cDNA amplification using PCR (Fig. 3.6). By adding a radioactive nucleotide in the reaction the cDNA can later be traced or visualized. The resulting amplicons, which theoretically, based on the oligo pairs used, correspond to all genes expressed by the cells, are electrophoresed through thin denaturing acrylamide gels that allow the separation of fragments with a single

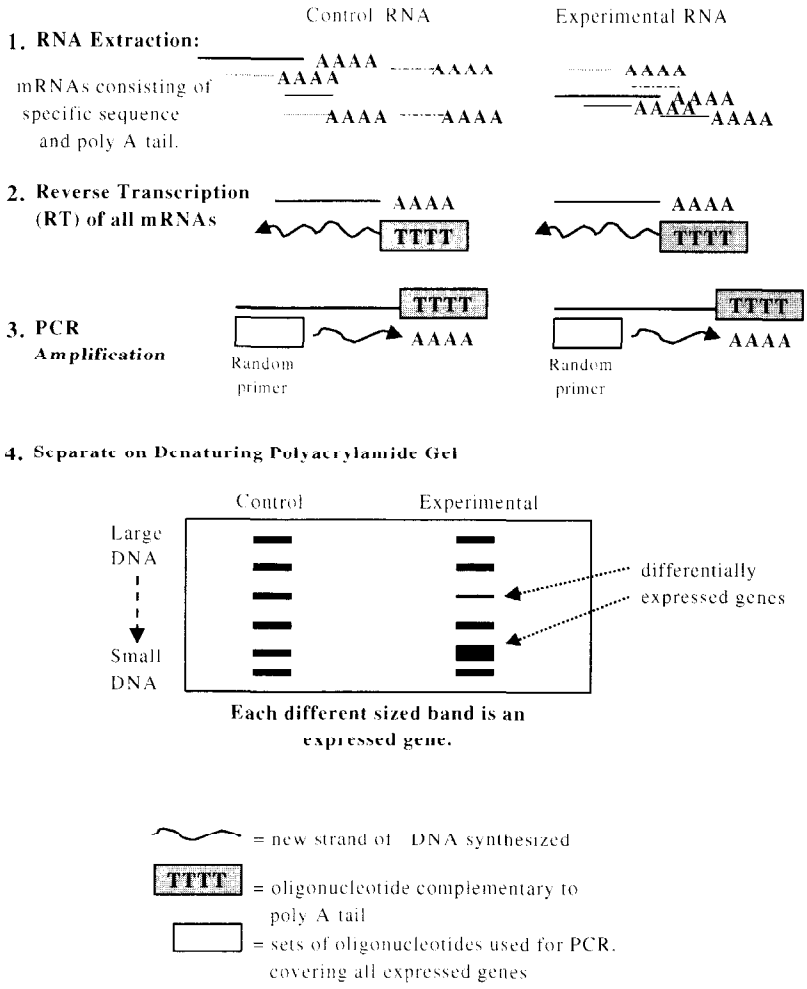

FIGURE 3.6 DD. A mixture of RNA from control and experimental material is subject to RT-PCR using random primers (short pieces of DNA) that can amplify all the genes expressed in a cell. A radioactive nucleotide is included (typically α ATP [S35]) so that the products of the reaction can be visualized after separation on acrylamide gels. The "bands" that show more or less intensity than the controls are, in theory, up- or downregulated, respectively. They are cut from the gel, reamplified, and used to (1) confirm their differential expression and (2) determine their DNA sequence to see if they are novel or previously reported to DNA databases such as GenBank.

base difference (DNA sequencing gels). By comparing the patterns of control and experimental samples, one can visualize distinct genes that are either upregulated (more intense) or downregulated (less intense) compared with control samples. For this reason, the method is called the "differential display" (DD). To identify the nature of the altered gene, the band is extracted from the gel, reamplified by PCR, and cloned for detailed sequence analysis. By consulting available public databases on the Internet (such as GenBank), one can determine if the gene is novel or, rather, previously isolated, characterized, and reported to the database. By using this approach several genes have been identified that are regulated by mechanical loading in rat bone, including osteopontin and myloperoxidase[22] and the glutamate/aspartate transporter.[23] This observation raises an interesting point about the technique: some findings are quite unexpected and lead to molecular pathways (in the latter case to the nervous system) that were probably not anticipated.

Other procedures can also be used to isolate genes that are differentially expressed in an experimental situation. One such alternative is "subtractive hybridization." There are many ways to carry out the method but all rely on the subtraction of genes common to the control and experimental mRNA. One recent report described the isolation of osteoclast-specific genes by subtracting cDNA, constructed from a rabbit osteoclast mRNA, with biotin-labeled mRNA previously isolated from rabbit spleen.[24] Thus, the cDNA in the osteoclast mRNA repertoire (called a library) that was also in the spleen was hybrized and subtracted out using a biotin-avidin method. The material that remained after subtraction was, in theory, osteoclast specific and is currently being analyzed.[24] By using the same procedure numerous genes were discovered that were differentially expressed in human cochlear (essential for hearing) mRNA compared with human brain mRNA. Some genes were previously identified such as collagens type I, II, and III while one was novel and termed Cock-5B2.[25] As with the differential display method, one must confirm that the gene is differentially regulated using Northern analysis, RPA, or quantitative RT-PCR. This confirmation eliminates "false positives" that appear to be differentially regulated in the first screen but really upon further analysis are not.

Still a third method for discovery of differentially expressed genes is called representational differences analysis (RDA). The procedure was originally designed[26] to measure the difference between two complex genomes (nuclear DNA) but was adapted so that genes could be isolated that encoded differentially expressed mRNA.[27] The method is PCR based and relies on a subtraction concept. In this procedure double-stranded DNA is made from the two RNA populations, and cut with a restriction enzyme so that it can be modified to contain specific DNA sequences at its terminus that can then be amplified using specific oligonucleotides. The DNA produced by the two different RNA preparations is referred to as "representative amplicons." The amplicon from the experimental sample is called the "tester" and the control material termed the "driver." By adding an excess of driver and oligos that can only amplify the tester to a mix of "driver and tester" the DNA common to the two samples forms a DNA duplex that cannot be amplified. Consequently, only tester DNA, not found in the driver, will be amplified and further characterized. The difference between this method and differential display (DD) is that in RDA only two samples can be compared (subtracted); in DD, numerous samples can be analyzed on a single gel. This is helpful for comparing patterns in different cell types or in one sample subject to different doses or time of treatment. In theory, DD, subtraction hybridization, and RDA will have the same outcome, namely, the isolation of genes differentially expressed between two samples. In this regard it is interesting to note that when the expression of genes was examined in rat livers treated with aflatoxin[28] using DD, RDA, and a subtraction procedure many genes were isolated that were differentially expressed; however, in each method different genes were obtained. No matter what method is selected, the isolated genes will need to be further characterized for functional capacity using the procedures outlined in later sections of this chapter (Sections 3.4 and 3.5) or in Chapter 4.

3.3.2 Serial Analysis of Gene Expression (SAGE)

In attempts to examine multitudes of genes simultaneously from a single sample, a nonradioactive modification of the DD procedure was devised.[29] The method relies on multiple amplification and cloning steps that result in the production of concatamers (many short pieces of DNA in tandem)

each corresponding to one molecule of RNA from the initial preparation. By extensive sequence analysis of the DNA, one can determine the occurrence (how many copies) and nature (known or unknown) of the most abundant genes in an mRNA sample. By using this gene discovery method,[29] numerous sets of identified and unidentified genes referred to as EST (expressed sequence tag) were submitted to gene databases that await further study to determine their structure and function. By analyzing over 300,000 transcripts derived from at least 45,000 different genes, Zhang et al.[30] discovered more than 500 genes that were expressed at different levels in normal and cancer cells. The advantage of this technique is that most of the equipment needed for the analysis would be found in a well-equipped molecular biology laboratory. Considering most of the analysis is based on DNA sequence comparison excellent computer software and hardware would additionally be required.

3.3.3 Microchip Technology (DNA Chip)

Along a similar rationale as the SAGE, the DNA chip was designed so that numerous genes could be analyzed simultaneously in a single RNA preparation.[31] The technique takes conventional arrays one step farther allowing the theoretical analysis of up to 10,000 genes in a single experiment. High throughput arrays use robots to spot the individual genes onto glass microscope slides resulting in the so-called cDNA chips. The mRNA extracted from the cells of interest is labeled with fluorescent dyes and is used as a probe. Special equipment has been developed that can detect and quantitate the intensity of hybridization results by measuring the intensity of the fluorescent signal. Two important points must be considered in this type of analysis, namely, (1) the characterization of the DNA that goes onto the chip and (2) the extensive "data mining" that follows. On the first point, before starting, one must have some knowledge about the composition of the DNA that will arrayed. For example, usually the array material is made by PCR of individual clones of a cDNA library (all the mRNAs converted into cDNA and packaged for biological expansion and immortalization). To avoid duplication of genes on the array, it is advantageous to have previously sequenced at least part of each DNA that will be used.[32] On the second point, copious amounts of data can be reamed from this microchip analysis. The difficult task, therefore, is to categorize the data in a meaningful fashion so that new hypotheses can be tested. Recently, the expression of 8600 distinct genes was examined in skin fibroblasts treated with serum. Interestingly, sets of genes were activated that previously have been linked to wound healing.[33] A good command of bioinformatics is required for this kind of analysis.

3.4 Gene Transfer

With the identification of genes involved in various genetic diseases, gene therapy is a new trend in the scientific medical field but is still in its experimental phase; for a review see Romano et al.[46] For example, in some diseases mutations of genes result in lack of protein production and theoretically the disease could be cured by transferring the normal gene into the diseased tissue.

From a more basic science point of view, once genes have been identified using the procedures described in the previous sections a critical next step is to determine the function of the gene. That is to say, if a gene is turned off, for example, by mechanical strain, it would be important to determine directly if an alteration in the expressed gene is in fact related to a functional consequence of the applied strain. One way to test the function of a target gene is to isolate the entire coding region (the part of the gene that will make a protein) and connect it to a regulatory DNA (promoter) that will force its expression or inactivation in a cell or tissue. Typical promoters that are used for such overexpression studies are derived from Simian Virus 40 (SV 40), cytomegalovirus (CMV), or rous sarcoma virus (RSV). The gene construct (transgene) is then transferred (transfected) into cultured cells (see Chapter 2) or used to make transgenic mice (see Chapter 4).

In both gene therapy and functional studies a critical step is the transfer of the gene of interest into the cells. Thus, the procedures discussed below are essential for (1) testing the function of a gene and (2) gene therapeutics.

3.4.1 Nonvirus-Based Gene Transfer

Numerous methods have been described for the transfer of genes into cells and tissues, and transfer efficiency depends on cell type, tissue origin, and species. Nonvirus-based methods may use basic polymers such as DEAE dextran[34] that have affinity for DNA or liposomes[35,36] that facilitate fusion and entry of the DNA through the cell membrane. Electroporation, on the other hand, relies on an electric field presumably to make small "holes" in the cell for DNA transfer.[37] Fine precipitates of $CaPO_4$ are also used and, in the authors' hands, are most successful for cultured bone cells.[38] Other novel procedures that might be suitable in the future for gene transfer, but are yet untested on skeletal tissue, include particle bombardment (gene gun)[39] and liposomes chemically tailored for mineralized tissue.[40] It should be pointed out that even "naked" DNA or DNA immersed in saline has also been successfully transferred into tissue. Recently, DNA encoding TGF-β1 was shown to suppress induced arthritis when injected intramuscularly into rodents.[41]

3.4.2 Adenovirus-Based Gene Transfer

Cells that are particularly resistant to gene transfer can often be successfully transfected using adenovirus-based methods. A major advantage of this method is that it is highly efficient and can be used on nondividing cells ranging from chick to human. One drawback is that the transgene expression is transient or temporary; the recombinant adenovirus is made replication deficient and, once infected into a cell, cannot make more of itself. Nevertheless this method is currently being explored for gene therapy in certain cases of arthritis.[42]

Recombinant adenovirus is made by genetically engineering the transgene into a plasmid DNA that is flanked with specific DNA sequences derived from the adenovirus. These virion sequences are complementary to sequences of a second larger piece of DNA that contains virion DNA that had these sequences removed (they are "missing"). By co-transfecting the two DNA types into 293 cells (these cells provide replication capacity) homologous recombination occurs in the cells (see Chapter 4) and the transgene is incorporated into the adenoviral genome providing the deleted virion with the "missing" material needed for viral production. In this way the transgene is recombined into the viral genome and can be propagated as virus particles. The presence of the transgene can be confirmed either by Northern, PCR, or by Western (protein) analysis using antibodies specific for the gene of interest and extracts from the infected cells (for details, see review by Becker et al.[43]). By growing virus in 293 cells, endless supplies can be produced to examine gene function in cultured cells and or injected tissues. Recently, the authors have tested a modification of this procedure that allows gene transfer without genetically engineering DNA into the adenoviral genome. Purified virus was covalently linked to polylysine and then incubated with the transgene. Efficient gene transfer was noted using either cultures of human bone marrow stroma (Fig. 3.7) and human trabecular bone[44] using this technique. A recent modification of the procedure has been devised[45] that substitutes polysine with polyethlyenimine (PEI) and may be an additional promising new adenovirus-based method that would also not require previous engineering of transgenes into the viral genome.

The adeno-associated virus (AAV) has, more recently, been of great interest for gene transfer;[46] it is distinct from adenovirus and has the advantage of integrating into the host genome. Because this virus requires a "helper" virus for replication, there are concerns that it could not be completely eliminated from viral preparations making it unsafe for clinical use. Nevertheless, as more becomes known about the vector system and viral life cycle, AAV holds promise for an alternative means of gene transfer and therapy in bone material.

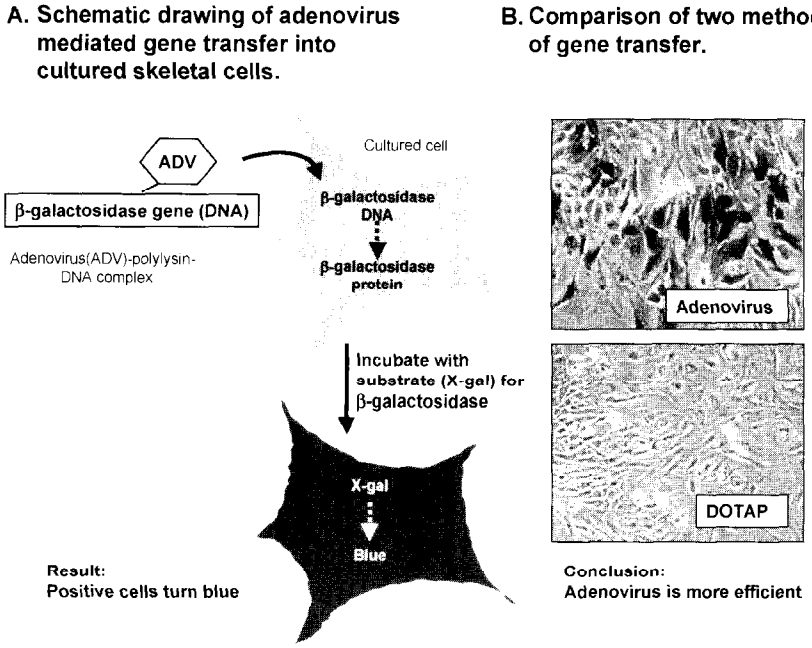

FIGURE 3.7 Gene transfer into cultured skeletal cells using two different methods. DNA for β-galactosidase is mixed with adenovirus previously covalently attached to polylysine (called a complex). The DNA (acidic) has affinity for the polylysine (basic). When DNA is added to the complex the adenovirus attaches to the cell membrane and carries the DNA into the cell like a "Trojan horse." β-Galactosidase mRNA is then transcribed from an attached promoter and the β-galactosidase mRNA translated into protein. Fixed cells incubated with the X-gal, which is a substrate for β-galactosidase, turn blue if it is present. The extent of the blue color is an indirect measure of the efficiency of gene transfer. The upper right panel shows the efficiency of gene transfer using the polylysine-adenovirus compared to DOTAP™ liposomes, which are shown on the lower right panel. The cultured cells receiving the DNA are marrow stromal fibroblasts derived from human bone.

3.4.3 Retrovirus-Based Gene Transfer

Retroviruses are RNA viruses that are constructed in a manner somewhat similar to adenoviruses.[47,48] Target genes are isolated and cloned into a vector that is flanked at each end by retrovirally derived long terminal repeats (LTR) that are essential for the life cycle of the virus. All other genes of the retrovirus have been previously removed to allow insertion of the foreign gene. To package the RNA inside a viral particle the DNA construct is transfected into special cells (usually NIH-3T3) that have been previously engineered to be able to package the foreign gene. Because gene transfer is often inefficient and many cells do not take up the transgene, a selectable marker such as neomycin resistance is included in the construct. In this way cells that do not take up the transgene and consequently the neomycin resistance gene are killed by treatment with neomycin. Surviving cells that produce the transgene, on the other hand, can be grown indefinitely and provide an endless source of packaged viral RNA. When recombinant retroviral particles are used to infect the tissue of interest, the transgene is converted to double-stranded DNA and incorporated into the host genome using the LTR sequences.[49] Thus, the major difference between the retrovirus and adenovirus is that the former is integrated in the host DNA and is therefore considered a "stable" transfection. The adenovirus transgene expression, while temporary or transient, does not require drug selection like the retrovirus. Retrovirus infection, on the other hand, requires cells that are undergoing cell division. Both methods suffer from the fact that the transgene, even when stably incorporated, is often not sustained. Nevertheless, for analysis of protein function either method could be used to examine fundamental parameters that occur within a reasonable time frame.

3.5 Inactivation of Specific Genes

3.5.1 Antisense: Oligonucleotides and Full-Length Constructs

One way to test for specific gene function is to ablate or inhibit the gene of interest and see what the ramifications are. This can be done by homologous recombination (see Chapter 4), which relies on double drug selection and vigorous cell division. Many bone cell types either do not divide well (the osteocyte) or do not respond well to drug treatment. For that reason, transient methods have been designed to interfere with RNA transcription from the promoter or with protein translation of the RNA. Small pieces of DNA are complementary to the target genes and are called antisense oligodeoxynucleotides (ODNS). In the first case, ODNS bind to the promoter and cause it to form a triplex structure that is no longer functional. In the second case, the ODNS bind to sequences in the mRNA, and through the action of RNase H present in the cell, the duplex is destroyed and can no longer function. In either case, both methods rely on efficient transfer of the ODNS into the target cells using the gene transfer methods described in Section 3.4 and reviewed in Ref.[50]

Antisense experiments have also been successful with full length cDNA (the entire coding sequence) engineered in a gene construct in the reverse orientation. In this way a longer antisense stretch of DNA is made that binds to the complementary mRNA and makes it inactive. One drawback to the latter approach is that selection of the transgene is required so that new cell lines can be derived. This is done so that the entire population (i.e., clone) of cells contains and expresses the transgene. Nevertheless using this approach Vander Molen et al.[51] created ROS 17/2.8 cells (see Chapter 2) that were unable to make connexin-43 because of the presence of an antisense transgene. Interestingly, these cells showed deficiencies in cell-to-cell coupling, indicating the importance of this protein in the networking of bone cells.

3.5.2 Ribozymes

Ribozymes are RNA molecules that can bind a target RNA and enzymatically cleave or cut it so it is no longer functional.[52] These unique RNAs are referred to as "hammerhead" because of their structure; they are composed of a hairpin loop (i.e., a stem loop where the stem contains complementary RNA) and have the potential to bind to target DNA with great specificity. Binding occurs through the "arms" of the hammerhead that are attached to the stem. As with the previously described procedures, the ribozyme must first be introduced into the cells by gene transfer for either transient expression or stable integration. Hammerhead ribozymes have recently been described that can distinguish a single base-pair difference between normal and type I collagen mutants.[53] This method offers promising new possibilities for inactivating mutated RNA that has a "dominant-negative" deleterious effect on bone such as occurs in osteogenesis imperfecta[54] or McCune Albright syndrome.[55] This and all of the other gene transfer methods can be taken to further steps of sophistication. By changing the promoter that directs the production of the transgene, one can elicit cell-specific or inducible expression repertoires so that the time and place of expression within a cell type or organism can be regulated (see Chapter 4).

3.6 Summary

In summary, there are many ways to examine molecular events in bone tissue. Each has advantages and disadvantages (Tables 3.1 and 3.2 for summary). Many methods are used in combination for confirmation of the data or for practical reasons such as limitation in sample size. Now that the genome project is complete all human genes have been sequenced. One hopes that this vast amount of new information can be used to examine sets of genes that are activated or inactivated during development, aging, and in the pathology of skeletal tissues. The ultimate challenge will be to determine how multiple genes and proteins work in concert to control the function and structure of bone material under normal and stressed conditions.

TABLE 3.1 Summary of Methods to Examine Molecular Events of Known Genes in Bone Tissue

	Number of Genes Detected	Advantage	Disadvantage
Northern blotting	One gene	• Direct quantitative detection of RNA	• Relatively large amounts of RNA necessary
RT-PCR	One gene	• Small amounts of RNA necessary • Nonradioactive	• Semiquantitative
RNase protection	Multiple genes: ~10	• Direct detection of RNA	• Radioactive
Array technology	Numerous genes: ~100–1000	• Screen many genes in one single experiment	• Differential gene expression needs confirmation using one of the methods above

TABLE 3.2 Summary of Methods for Gene Discovery

	Description	Advantage	Disadvantage
Differential display	First method described to screen many known and novel genes simultaneously	• First steps easy • Many samples on a single gel (experiment)	• Analysis of clones is lengthy • False positives
SAGE	Nonradioactive method of screening many known and novel genes	• Nonradioactive • Screen many genes in one cloning step • Quantitation possible • Modification of the technique prevents nonspecific binding of linkers	• Extensive computer analysis required • Multiple cloning steps involved
RDA, subtractive hybridization	Subtract common genes by hybridization of control to experimental	• Differentially expressed genes obtained directly	• Only two samples can be compared at one time
Microchip technology	Fluroscence-based method of screening many known and novel genes	• Nonradioactive • Fluorescent quantitation	• Extensive characterization of cDNA library is needed prior to designing the chip • Extensive data mining

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